DOCKET NO.: 279737US0PCT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

IN RE APPLICATION OF: Giuseppe ARPAIA, et al.

SERIAL NO.: NEW U.S. PCT APPLICATION

FILED: HEREWITH

INTERNATIONAL APPLICATION NO.: PCT/EP04/05401

INTERNATIONAL FILING DATE: May 18, 2004

FOR: METHOD OF CHROMATOGRAPHIC ANALYSIS OF A PROTEIN SOLUTION

REQUEST FOR PRIORITY UNDER 35 U.S.C. 119
AND THE INTERNATIONAL CONVENTION

Commissioner for Patents
Alexandria, Virginia 22313

Sir:

In the matter of the above-identified application for patent, notice is hereby given that the applicant claims as priority:

COUNTRY	APPLICATION NO	DAY/MONTH/YEAR
EPC	03101458.2	21 May 2003

Certified copies of the corresponding Convention application(s) were submitted to the International Bureau in PCT Application No. PCT/EP04/05401. Receipt of the certified copy(s) by the International Bureau in a timely manner under PCT Rule 17.1(a) has been acknowledged as evidenced by the attached PCT/IB/304.

Respectfully submitted,
OBLON, SPIVAK, McCLELLAND,
MAIER & NEUSTADT, P.C.

Norman F. Oblon
Attorney of Record
Registration No. 24,618
Surinder Sachar
Registration No. 34,423
Corwin P. Umbach, Ph.D.
Registration No. 40,211

Customer Number

22850

(703) 413-3000
Fax No. (703) 413-2220
(OSMMN 08/03)

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Anmeldung Nr:
Application no.: 03101458.2
Demande no:

Anmeldetag:
Date of filing: 21.05.03
Date de dépôt:

Anmelder/Applicant(s)/Demandeur(s):

ARES TRADING S.A.
Chateau de Vaumarcus
CH-2028 Vaumarcus
SUISSE

Bezeichnung der Erfindung/Title of the invention/Titre de l'invention:
(Falls die Bezeichnung der Erfindung nicht angegeben ist, siehe Beschreibung.
If no title is shown please refer to the description.
Si aucun titre n'est indiqué se referer à la description.)

Improvements in analytical methods

In Anspruch genommene Priorität(en) / Priority(ies) claimed /Priorité(s)
revendiquée(s)
Staat/Tag/Aktenzeichen/State/Date/File no./Pays/Date/Numéro de dépôt:

Internationale Patentklassifikation/International Patent Classification/
Classification internationale des brevets:

C07K1/00

Am Anmeldetag benannte Vertragstaaten/Contracting states designated at date of
filing/Etats contractants désignées lors du dépôt:

AT BE BG CH CY CZ DE DK EE ES FI FR GB GR HU IE IT LU MC NL
PT RO SE SI SK TR LI

IMPROVEMENTS IN ANALYTICAL METHODS

This invention relates to improved methods for the analysis of proteins.

5 More specifically, it relates to the analysis of proteins by HPLC methods. By "analysis" of proteins is meant here both the quantitative determination and the assessment of purity of a protein.

In these methods, the protein must be dissolved in a an aqueous solvent and
10 diluted to an extent acceptable for the chromatographic system used. During handling of the aqueous protein solution the risk exists that part of the protein is lost by adsorption to handling and containment equipment, such as glass or plastic walls of capillaries, test-tubes, beakers, syringes, etc., making quantitation of protein difficult.

15

It has now been discovered that a surfactant of the class of Poloxamers does not interfere with the chromatographic analysis while at the same time avoiding losses of protein.

20 Poloxamers are block copolymers made of poly(oxyethylene)- poly(oxypropylene) blocks with M.W. ranges from 1,000 to > 16,000. Their main characteristic is that the poly(oxyethylene) segments are hydrophobic and the poly(oxypropylene) segments hydrophilic. These substances behave as non-ionic surfactants and are generally known with the commercial name
25 "Pluronics".

Many grades of Pluronics at various Molecular Weight and concentration ranges can be used in accordance with this invention.

30 As mentioned above, the Poloxamer (Pluronic) surfactants are block copolymers of ethylene oxide (EO) and propylene oxide (PO). The propylene oxide block (PO) is sandwiched between two ethylene oxide (EO) blocks.

$$HO-(CH_2CH_2O)_x\text{-}(CH_2\overset{\overset{\displaystyle CH_3}{|}}{C}HO)_y\text{-}(CH_2CH_2O)_x\text{-}H$$

EO	PO	EO
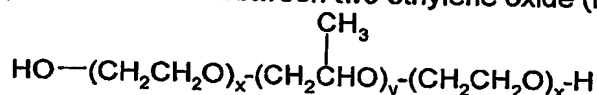
35 Pluronic surfactants are synthesised in a two-step process:

1. A hydrophobe of the desired molecular weight is created by the controlled addition of propylene oxide to the two hydroxyl groups of propylene glycol; and

40 2. Ethylene oxide is added to sandwich the hydrophobe between hydrophilic groups.

In Pluronic® F77, the percentage of polyoxyethylene (hydrophile) is 70%, and the molecular weight of the hydrophobe (polyoxypropylene) is approximately 2,306 Da.

In Pluronic F87, the percentage of polyoxyethylene (hydrophile) is 70%, and the molecular weight of the hydrophobe (polyoxypropylene) is approximately 2,644 Da.

In Pluronic F88, the percentage of polyoxyethylene (hydrophile) is 80%, and the molecular weight of the hydrophobe (polyoxypropylene) is approximately 2,644 Da.

In Pluronic F68, the percentage of polyoxyethylene (hydrophile) is 80%, and the molecular weight of the hydrophobe (polyoxypropylene) is approximately 1,967 Da.

Typical properties of Pluronic F77 are listed below:
Average Molecular Weight: 6600;
Melt/pour point: 48°C ;
Physical Form @ 20°C : solid;
Viscosity (Brookfield) cps: 480 [liquids at 25°C, pastes at 60°C and solids at 77°C];
Surface tension, dynes/cm @ 25°C;
     0.1% Conc. : 47.0
     0.01% Conc. : 49.3
     0.001% Conc.: 52.8
Interfacial tension, dynes/cm @ 25°C vs. Nujol;
     0.1% Conc. : 17.7
     0.01% Conc. : 20.8
     0.01% Conc. : 25.5
Draves Wetting, Seconds 25°C
     1.0% Conc.: > 360
     0.1% Conc.: > 360
Foam Height
     Ross Miles, 0.1%, mm @ 50°C: 100
     Ross Miles, 0.1%, mm @ 26°C: 47
     Dynamic, 0.1%, mm @ 400 ml/min: > 600
Cloud point in aqueous solution, °C
     1% Conc.: >100
     10% Conc.: >100
HLB (hydrophile-lipophile balance): 25

Typical properties of Pluronic F87 are listed below:
Average Molecular Weight: 7700;
Melt/pour point: 49°C ;
Physical Form @ 20°C : solid;
Viscosity (Brookfield) cps: 700 [liquids at 25°C, pastes at 60°C and solids at 77°C];
Surface tension, dynes/cm @ 25°C;
     0.1% Conc. : 44.0
     0.01% Conc. : 47.0
     0.001% Conc.: 50.2
Interfacial tension, dynes/cm @ 25°C vs Nujol;

0.1% Conc. : 17.4
0.01% Conc. : 20.3
0.01% Conc. : 23.3
Draves Wetting, Seconds 25°C
1.0% Conc.: > 360
0.1% Conc.: > 360
Foam Height
Ross Miles, 0.1%, mm @ 50°C: 80
Ross Miles, 0.1%, mm @ 26°C: 37
Dynamic, 0.1%, mm @ 400 ml/min: > 600
Cloud point in aqueous solution, °C
1% Conc.: >100
10% Conc.: >100
HLB (hydrophile-lipophile balance): 24

Typical properties of Pluronic F88 are listed below:
Average Molecular Weight: 11400;
Melt/pour point: 54°C ;
Physical Form @ 20°C : solid;
Viscosity (Brookfield) cps: 2300 [liquids at 25°C, pastes at 60°C and solids at 77°C];
Surface tension, dynes/cm @ 25°C;
0.1% Conc. : 48.5
0.01% Conc. : 52.6
0.001% Conc.: 55.7
Interfacial tension, dynes/cm @ 25°C vs Nujol;
0.1% Conc. : 20.5
0.01% Conc. : 23.3
0.01% Conc. : 27.0
Draves Wetting, Seconds 25°C
1.0% Conc.: > 360
0.1% Conc.: > 360
Foam Height
Ross Miles, 0.1%, mm @ 50°C: 80
Ross Miles, 0.1%, mm @ 26°C: 37
Dynamic, 0.1%, mm @ 400 ml/min: > 600
Cloud point in aqueous solution, °C
1% Conc.: >100
10% Conc.: >100
HLB (hydrophile-lipophile balance): 28

Typical properties of Pluronic F68 are listed below:
Average Molecular Weight: 8400;
Melt/pour point: 52°C ;
Physical Form @ 20°C : solid;
Viscosity (Brookfield) cps: 1000 [liquids at 25°C, pastes at 60°C and solids at 77°C];
Surface tension, dynes/cm @ 25°C;
0.1% Conc. : 50.3
0.01% Conc. : 51.2

0.001% Conc.: 53.6

Interfacial tension, dynes/cm @ 25°C vs Nujol;

    0.1% Conc. : 19.8

    0.01% Conc. : 24.0

    0.01% Conc. : 26.0

Draves Wetting, Seconds 25°C

    1.0% Conc.: > 360

    0.1% Conc.: > 360

Foam Height

    Ross Miles, 0.1%, mm @ 50°C: 35

    Ross Miles, 0.1%, mm @ 26°C: 40

    Dynamic, 0.1%, mm @ 400 ml/min: > 600

Cloud point in aqueous solution, °C

    1% Conc.: >100

    10% Conc.: >100

HLB (hydrophile-lipophile balance): 29

Other polymers having properties similar to those listed above may also be used in the methods of the invention. The preferred surfactant is Pluronic F68 (Poloxamer 188), and surfactants having similar properties.

Therefore, this invention relates to an improved method of chromatographic analysis of a protein including the step of preparation of a protein sample with a protein concentration acceptable for the chromatographic system used, the improvement consisting in adding a Poloxamer to the protein sample solution.

The method can be used essentially with any protein, for example insulin, etanercept, Factor VIII, growth hormone, antibodies (such as infliximab), In a preferred embodiment, the protein is a glycoprotein, such as erythropoietin (EPO), darbepoietin alfa, human protein C, interferons (such as interferon beta 1a, 1b), alpha galactosidase A, Preferably the protein is a dimeric protein (including heterodimeric), particularly preferably a dimeric glycoprotein, for example thyroid-stimulating hormone (TSH), and the gonadotrophins: follicle-stimulating hormone (FSH), luteinising hormone (LH), and chorionic gonadotrophin (CG).

In the following Examples which follow, Pluronic F68 (Poloxamer 188) is used at the concentration of 100 μg /ml in ultra-pure water. It is however understood that the use of different grades of Pluronic and different concentrations of the same is encompassed by the present invention.

<u>EXAMPLE1</u>

<u>Improved sample preparation in the SEC method of quantitative determination of FSH</u>

The purpose of this study was to qualify an improved sample preparation in the SEC method for protein content in a preparation containing rec-FSH (Fertinex, in this case Fertinex 75 IU).

The modification implemented was the use of a solution including 100 µg/ml Pluronic F68 in ultra-pure water for preparation of all protein solutions (sample, control sample and standard) in order to control losses of protein due to adsorption.

5   This study shows that a single point calibration curve using drug product reference standard is suitable to determine protein content with a good total precision of 2.0% and that the method is linear within the range tested (26.6 to 160 µg/ml).

10 In addition, both Waters and Varian HPLC systems can be used as shown during the study. The difference in the mean protein content results of all batches is lower than the total precision of the method.

It is important to emphasize that this change did not have any impact on the original SEC method itself.

15 More specifically, the modification of the sample and standard preparations were as follows:

- Use of a 100 µg/ml Pluronic F68 in ultra-pure water for the preparation of the samples and reference standard.

- Blank solution used through the analytical sequence is the 100 µg/ml Pluronic F68 solution.

20 - The preparation for SST control sample as well as control samples injected over the analytical session are performed by reconstituting and pooling enough ampoules to allow the injections of the system suitability as well as for the controls which are injected throughout the analytical session.

25

The precision of an analytical method expresses the closeness of agreement (degree of scatter) between a series of measurements obtained from multiple sampling of the same homogeneous sample under the prescribed conditions. Precision may be considered at three levels :

30 repeatability (or intra-assay precision), intermediate precision and reproducibility. During this study, repeatability, intermediate precision as well as reproducibility of the assay were addressed.

In five independent analytical sessions, three Fertinex drug products batches were quantified against the standard (calibration curve).

35 With the results of Fertinex 75 IU presented in Table 1, an analysis of variance (ANOVA) Nested design was used to estimate the repeatability (or intra-assay precision), intermediate precision and reproducibility (total precision) of the analytical method. The total number of results under study was 45. The results obtained were as follows:

40 - Repeatability (or intra-assay precision): 1.3%

- Intermediate precision: 1.0 %

- Reproducibility (total precision): 1.6%

Overall results are tabulated in the following Table 1:

Table 1: Protein content results (µg/ampoule) obtained with calibration curve standard

Batch number	Run 1	Run 2	Run 3	Run 4	Run 5
17301010	6,41	6,42	6,32	6,56	6,41
17315040	6,13	6,06	5,94	6,11	6,15
17318040	5,54	5,58	5,49	5,59	5,51
Control sample	6,00	6,18	6,04	6,21	6,07

The standard results obtained during the precision and ruggedness studied were used to address the linearity and range of the analytical method. The approach for the statistical analysis was to check variance homogeneity (Cochran C and Barlett tests), lack of fit and perform regression analysis (correlation coefficient). Run 1 to Run 5 were performed with a Waters systems whereas Run 6 to Run 8 were performed with a Varian system.

Table 2: Statistical results for linearity

	Run 1	Run 2	Run 3	Run 4	Run 5	Run 6	Run 7	Run 8
Cochran's C test	0,99	0,98	0,99	0,99	0,99	0,98	0,96	0,98
Barlett's test	0,99	0,98	0,99	0,99	0,99	0,98	0,96	0,98
Lack of fit	0,97	0,18	0,83	0,92	0,81	0,89	0,03	0,07
Slope	1,0058	0,9926	1,0028	1,0066	0,9940	0,9903	1,0202	0,9888
Intercept	6,1557	6,2238	6,1574	6,2099	6,2249	5,5055	5,7437	5,5214
Correlation coeff.	1,0000	0,9999	0,9999	0,9999	1,0000	0,9999	0,9998	0,9999

As can be seen, the p-values of Cochran C and Barlett tests are always greater than 0.05 which means that there is no statistically significant difference amongst the standard deviations at 95% confidence level and a correlation coefficient higher than 0.9980 is always met.

Lack of fit to determine whether linear regression is an adequate model to describe the observed data was performed. Based on this statistical test, linear regression resulted to be the best model to describe the data even if the p-value of Run 7 is not greater than 0.05. For that particular analytical session, the linear regression is still the best model with a 99.96% fit. Other models, such as square root and exponential models were tested and did not show a better fit of the data observed.

EXAMPLE 2

Improved sample preparation and spiking procedure in the SEC method of purity determination of FSH

The modification implemented in the analytical method is the use of a solution including 100 µg/ml Pluronic F68 in ultra-pure water for preparation of all protein solutions (sample, spike solution) to control losses of protein due to adsorption. The modifications were made specifically to increase the precision of the method, without impacting the chromatography of the method, to enable a more precise and accurate purity determination.

In the frame of this study, determination of the precision of the method was also investigated. The precision of the method (Total precision 1.8%) was slightly improved when compared to the precision of the method observed during validation of the analytical method without introduction of Pluronic F68 (Precision 2%). Routine recovery of the spike solutions at 100% indicates a good accuracy.

An additional spiking experiment was performed to further determine accuracy of the method. It was observed that different surface is obtained for the spiking solution with and without Pluronic F68. The area of the spiking solution with Pluronic F68 in the sample preparation is approximately two times greater than the spiking solution prepared without the use of Pluronic F68.

It is believed that the different areas observed are due to adsorption of the dissociated sub-units on the polypropylene material used for sample preparation.

It can be seen in the following Tables 3 and 4 that the recovery of spiked free sub-units is within the range 95% - 105%. In addition it can also be seen that the CV% over the five runs are ranging from 1.0% to 1.5% for both purity and recovery of spike.

Table 3: %Recovery of spike results

Batch#	Run 1	Run 2	Run 3	Run 4	Run 5	Mean	CV %
17343109	98,0%	98,1%	100,3%	99,6%	99,4%	99,1%	1,0%
17349129	98,9%	99,6%	102,1%	100,7%	102,2%	100,7%	1,5%
17301010	99,8%	100,7%	102,0%	99,2%	99,8%	100,3%	1,1%
17304010	99,8%	102,4%	101,6%	101,0%	99,9%	100,9%	1,1%

Table 4: %Purity of spike results

Batch#	Run 1	Run 2	Run 3	Run 4	Run 5	Mean	CV %
17343109	97,0%	94,4%	93,9%	96,3%	96,3%	95,6%	1,4%
17349129	96,2%	93,4%	93,3%	95,5%	94,4%	94,6%	1,4%
17301010	96,8%	95,0%	94,1%	96,9%	97,2%	96,0%	1,4%
17304010	95,9%	93,6%	94,0%	95,9%	97,0%	95,3%	1,5%

In the frame of another study protocol, the analysis of drug product batches with and without the use of Pluronic F68 was performed. One of the results is that the area of the spiking solution (dissociated r-hFSH) is approximately multiplied by two with the introduction of Pluronic F68. This phenomenon is most probably due to adsorption of free sub-units on the material used during sample preparation if Pluronic F68 is not present. To determine if this increase of area has an impact on the analysis, a spiking experiment at three levels without and with Pluronic F68 was performed. The following samples were tested in duplicate:

1.Sample preparation with Pluronic F68

Sample without spiking solution;

Sample spiked with dissociated r-hFSH (2 µg by injection);

Spiking solution (2 µg dissociated r-hFSH by injection);

Sample spiked with dissociated r-hFSH (1.5 µg by injection ;
Spiking solution (1.5 µg dissociated r-hFSH by injection);

Sample spiked with dissociated r-hFSH (1 µg by injection);

Spiking solution (1µg dissociated r-FSH by injection).

The results are presented in table 5:

Table 5: Results of sample preparations using Pluronic F68

Spiking level	Area of spiking solution	% Recovery of areas	% Purity	% Recovery of spike
100% (2 µg/inj)	2037915	N/A	95%	101%
75% (1.5 µg/inj)	1545486	76%	94%	101%
50% (1 µg/inj)	1001055	49%	94%	102%

From the above table, it can be seen that the addition of Pluronic F68 gives good results in term of % recovery of spike, being close to the theoretical recovery (100%) and % Recovery of areas. This latter parameter is calculated by dividing the area of the spiking solution at the defined spiking level by the area of the spiking solution at 100%. The result (i.e. 76% for spiking with 1.5 µg/injection) is compared with the theoretical spiking level performed (i.e. 75%).

2.   Sample preparation without Pluronic F68

Sample without spiking solution;

Sample spiked with dissociated r-hFSH (2 µg by injection);

Spiking solution (2 µg dissociated r-hFSH by injection);

Sample spiked with dissociated r-hFSH (3 µg by injection) ;

Spiking solution (3 µg dissociated r-hFSH by injection);

Sample spiked with dissociated r-hFSH (4 µg by injection);

Spiking solution (4 µg dissociated r-hFSH by injection)

Results are shown in the following Table 6:

Table 6 : Results of sample preparations without Pluronic F68

Spiking level	Area of spiking solution	% Recovery of areas	% Purity	% Recovery
100% (2 µg/inj)	922106	N/A	90%	105%
150% (3 µg/inj)	1744753	189%	89%	104%
200% (4 µg/inj)	2702520	292%	89%	104%

For the results obtained without the use of Pluronic F68 in the sample preparation, the recovery of the spike solution deviates 4-5% from the theoretical recovery (100%). For % recovery of areas, the results deviate significantly from the theoretical spiking level performed, due to a too low area of the spike solution.

It is believed that the different results obtained with and without Pluronic F68 can be explained by the adsorption of the test samples on the material used during sample preparation. The area of spiking solution obtained for the same spiking level (100%) is approximately two times greater when Pluronic F68 is present and avoids the adsorption.

The adsorption of free sub-units can be calculated by the difference between the area of the spiking solution with Pluronic F68 and the area of the spiking solution without Pluronic F68 at 2 µg per injection (100%) spiking level. The difference corresponds to an area of 1'115'809. This area reflects the amount of dissociated sub-units adsorbed during sample preparation and can be used to correct the area obtained in absence of Pluronic F68. The recoveries of area calculated taking into account this correction can be seen in table 7. The data are closer to the theoretical spiking level.

Table 7: %Recovery of area with and without taking into account adsorption

Spiking level	%Recovery of areas taking into account adsorption	
	No	Yes
3 µg/inj (150%)	189%	140%
4 µg/inj (200%)	292%	187%

The purity results presented in tables 5 and 6 differ without and with the introduction of Pluronic. This difference is also believed to be an effect of adsorption phenomenon.

Several product batches were tested by SEC for purity without and with introduction of Pluronic F68 in sample preparation. The results can be seen in table 8:

Table 8: %Recovery and %Purity results of drug product tested without and with Pluronic F68

Batch#	Without Pluronic		With Pluronic	
	%Recovery	%Purity	%Recovery	%Purity
17353066	98%	89%	96%	94%
17303019	101%	91%	100%	97%
17306019	104%	91%	97%	98%
17309029	100%	90%	100%	97%
17323049	101%	92%	98%	97%
17324049	98%	94%	97%	99%
17340089	100%	93%	99%	97%
17341089	101%	91%	98%	98%
17345119	100%	90%	100%	96%
17305019	105%	88%	99%	101%
17315039	99%	91%	101%	95%
17302019	100%	87%	99%	96%
17501019	106%	86%	99%	96%
Mean	101,0%	90,2%	98,7%	97,0%
Standard deviation	2,5%	2,3%	1,4%	1,8%
CV %	2,5%	2,5%	1,5%	1,8%

Purity and recovery of spiking solution

Based on the data presented in Table 8 above, it can be seen that the difference between the recovery means without and with introduction of Pluronic is about 2%. This difference is statistically significant when an ANOVA at 95% confidence level is performed (p-value 0.008). In addition, when looking at the same table one can also see a statistically significant difference of approximately 7% in purity with and without introduction of Pluronic F68 (ANOVA p-value $1.2 \ 10^{-8}$).

These statistical differences in purity and recovery of spiking solution can be explained by adsorption phenomenon which occurs during sample preparation (see the following Table 9):

Table 9 : Area variability

Batch #	Total area of FSH spiking solution				Total area of unspiked sample				Dimers and aggregates area of sample			
	Area		CV%		Area		CV%		Area		CV%	
Pluronic	No	Yes	No	Yes	No	Yes	No	Yes	No	Yes	No	Yes
173353066	1409832	2658545	2,2	1,7	7377193	7767243	0,4	2,5	85841	90638	10,33	3,92
173303019	1438014	2513326	0,5	0,2	6136762	6312492	4,8	2,2	65913	64896	2,28	3,05
173306019	1438014	2513326	0,5	0,2	6029360	6474462	1,2	1,2	69387	65633	3,06	6,49
173309029	1121632	2380100	3,1	2,4	6554845	6906608	0,5	0,5	74694	83950	4,36	5,06
173323049	1300382	2429203	1,2	1,5	5692277	6157225	1,8	1,5	64801	67053	5,47	7,62
173324049	1300382	2429203	1,2	1,5	5856669	6129612	4,6	1,5	61088	58535	5,49	0,72
173340089	1449322	2564021	2,3	3,4	6316392	6577138	1,7	0,7	55831	64916	1,93	3,53
173341089	1449322	2564021	2,3	3,4	6311904	6658876	1,3	0,4	51410	66879	6,79	2,36
173345119	1121632	2380100	3,1	2,4	5678718	6094436	0,5	0,3	61319	71681	5,86	0,98
173305019	1416915	2609854	3,5	0,8	6105716	6366397	3,4	1,3	81412	76271	8,77	1,96
173315039	1207140	2528909	7,4	5,7	6629111	7129130	1,4	0,6	69514	65511	1,74	3,77
173302019	1207140	2528909	7,4	5,7	6306795	6975041	3,8	0,6	97979	94241	5,28	8,59
175010119	1416915	2609854	3,5	0,8	7416153	7580193	1,1	2,1	69441	67857	6,4	6,1
Mean	1328972	2516105	2,9	2,3	6339377	6702219	2,0	1,2	69895	72159	5,2	4,2
CV %	9,5%	3,5%	76%	81%	8,7%	8,1%	77%	63%	18%	15%	50%	59%

Table 9 : Area variability (continued)

Batch #	Dissociated FSH area of spiked sample				Total area of spiked sample			
	Area		CV%		Area		CV%	
Pluronic	No	Yes	No	Yes	No	Yes	No	Yes
17353066	2132665	3052448	2,5	1,08	8618890	9989197	0,92	0,41
17303019	1908454	2640090	4,5	0,43	7634720	8866597	1,52	0,83
17306019	1920007	2564678	1,2	0,81	7739564	8723677	1,12	0,58
17309029	1697129	2520633	6,0	2,01	7695502	9270545	2,16	1,48
17323049	1697821	2542802	1,8	1,14	7033218	8410312	1,14	1,09
17324049	1596022	2439971	6,4	2,61	7022864	8324957	2,32	0,81
17340089	1862023	2678583	3,2	1,27	7763745	9078480	0,79	1,49
17341089	1977742	2642383	1,3	1,16	7846421	9034549	0,31	0,27
17345119	1616907	2555312	5,7	1,80	6808816	8480285	1,21	1,17
17305019	2044400	2479940	1,2	2,99	7863722	8851837	3,27	0,95
17315039	1722445	2785597	5,0	0,08	7721964	9750217	2,01	0,41
17302019	1943343	2681371	11,0	2,72	7543478	9363087	2,68	2,07
17501019	2354166	2847934	0,4	0,5	9364548	10138501	0,2	0,35
Mean	1882548	2648596	3,9	1,4	7742881	9098634	1,5	0,9
CV %	12%	6,3%	77%	65%	8,6%	6,4%	61%	59%

The precision (CV%) of the areas used for the purity and recovery of spiking solutions calculation is always better with Pluronic F68 than without the introduction of Pluronic F68.

The adsorption rate of dimers and aggregates, free sub-units and monomer are different as one can see when calculating the ratio mean area with Pluronic F68/ mean area without Pluronic F68. As can be seen in table 10, the %Area increase with Pluronic F68 is not constant depending on the area considered.

Table10: %Area increase with introduction of Pluronic F68

Area considered	%Area increase
Total area of spiking solution	6%
Total area of unspiked sample	89%
Dimers and aggregates areas of unspiked sample	41%
Dissociated sub-units area of spiked sample	3%
Total area of spiked sample	18%

To determine purity, spiking of free sub-units must be performed due to the fact that the free sub-units peak is not resolved from main peak during testing of sample preparation. The %Purity (or %Monomer) is expressed by the formula below:

$$\%Purity = \frac{A + B - C - D}{A}$$

Where: A : Total area of sample without spike
B : Total area of spiking solution
C : Sub-units peak area of sample spiked
D : Dimers and aggregates in sample without spike

The acceptance criteria for recovery of spike is expressed as follows:

$$\%Recovery = \frac{E}{A + B}$$

Where: A : Total area of sample without spike
       B : Total area of spiking solution
       E : Total area of spiked sample

Taking the above formulas into consideration, calculation of purity with and without introduction of Pluronic F68 can be performed. In addition, to take into consideration the effect of Pluronic F68, calculations can also be done with areas without introduction of Pluronic F68 taking into account the adsorption effect. The formulas will be the following :

$$\%\text{Purity corrected} = \frac{A \times Ra + B \times Rb - C \times Rc - D \times Rd}{A \times Ra}$$

$$\%\text{Recovery corrected} = \frac{E \times Re}{A \times Ra + B \times Rb}$$

Where: A : Total area of sample without spike
       B : Total area of spiking solution
       C : Sub-units peak area of sample spiked
       D : Dimers and aggregates in sample without spike
       E : Total area of spiked sample
       Rx: Ratio mean area X with Pluronic F68 divided by mean area X without Pluronic F68.

EXAMPLE 3

Interferon beta-1a assay by RP-HPLC: Qualification of a One standard point approach versus a standard curve approach.

This Example shows how, in the case of Interferon beta-1a assay by RP-HPLC, it was possible to modify a standard curve approach, hereinafter referred to as "current assay", to a One Standard Point approach by applying the improved method of this invention, that is, by using a Pluronic surfactant to avoid sample losses.

In the drug substance sample preparation for the improved method of the invention, Interferon beta-1a was diluted 1 to 7 using as the dilution buffer 0.05M sodium acetate containing 0.1% Poloxamer 188 (Pluronic F68) at pH 3.8 instead of 0.05M sodium acetate at pH 3.8 without Poloxamer.

The optimized RP-HPLC assay was qualified according to the ICH Guidelines and the following characteristics were addressed:

• Range of linearity: The linearity was verified for the injection of Interferon-beta-1a in the range of 3.3 to 8.8 μg, with an intercept statistically not different from zero. These results support the One Standard Point approach.

• <u>Precision:</u> The precision of the optimized assay using the Drug Product Standard and the One Standard Point approach was compared to the precision obtained with the current assay using the Drug Substance Standard and the Standard curve approach. The overall precision obtained for one

5   replicate with the optimized assay (pooled CV of 1.2 % for all Drug Substances and Drug Products samples) was shown to be better than the precision of 1.9 % obtained for one replicate with the current assay.

• <u>Specificity:</u> the components that can be observed in the optimized assay are
10   already present in the Drug Substance and Drug Product formulations analyzed in the current assay which was validated for specificity. The presence of Poloxamer 188 in the new Standard was demonstrated to not interfere in the optimized assay. As no additional components are introduced in the optimized assay, the optimized assay is therefore specific.

15

• <u>Accuracy:</u> The accuracy of the optimized assay was assessed by comparison of the results obtained with the optimized assay versus the results obtained with current assay, these last results being considered as the reference value. For this purpose, several Drug Substances and Drug

20   Products (Liquid formulation containing HSA, Liquid formulation HSA free) were analyzed. In all cases, the ratio Optimized / Current Interferon-beta-1a assay was not statistically different from 100%, which demonstrates that the two Interferon beta-1a assays generate statistically equivalent results.

25   In conclusion, the optimized assay was qualified. The method has a better precision than the current assay, and generates accurate results statistically equivalent to those obtained with the current assay.

This invention has been described with reference to examples of both quantitative determination of proteins and purity assessment of the same in
30   both SEC and RP-HPLC chromatographic methods.

It will be apparent that many other equivalent examples can be done without departing from the spirit and scope of the invention that is defined by the following claims.

CLAIMS

1. In a method of chromatographic analysis of a protein sample solution, the improvement consisting in adding a Poloxamer to the protein sample solution.

2. In a method of chromatographic analysis of a protein including the step of preparing a diluted sample for bringing the protein concentration to a level acceptable for the chromatographic system used, the improvement consisting in adding a Poloxamer to the diluted sample solution.

3. The improved method of claim 1 or 2, wherein the chromatographic analysis is the quantitative determination of protein content.

4. The improved method of claim 1 or 2, wherein the chromatographic analysis is the assessment of protein purity.

5. The improved method of any of the preceding claims wherein the chromatography is size-exclusion chromatography (SEC) or reverse-phase HPLC (RP-HPLC).

6. The improved method of any of the preceding claims wherein the protein on which analysis is carried out is FSH.

7. The improved method of any of the preceding claims wherein the protein on which analysis is carried out is Interferon beta-1a.

8. The improved method of any of the preceding claims wherein the Poloxamer is Pluronic F68 (Poloxamer 188).

9. The improved method of claim 8 wherein Pluronic F68 is employed at a concentration of 100 µg/ml in ultra-pure water in the protein sample solution.

10. The improved method of claim 8 wherein Pluronic F68 is employed at a concentration of 0.1% in sodium acetate buffer at pH 3.8 in the protein sample solution.

Abstract

An improved method of chromatographic analysis of protein samples is disclosed in which a Pluronic surfactant is used.